Reconstitution of the 26S proteasome reveals functional asymmetries in its AAA+ unfoldase

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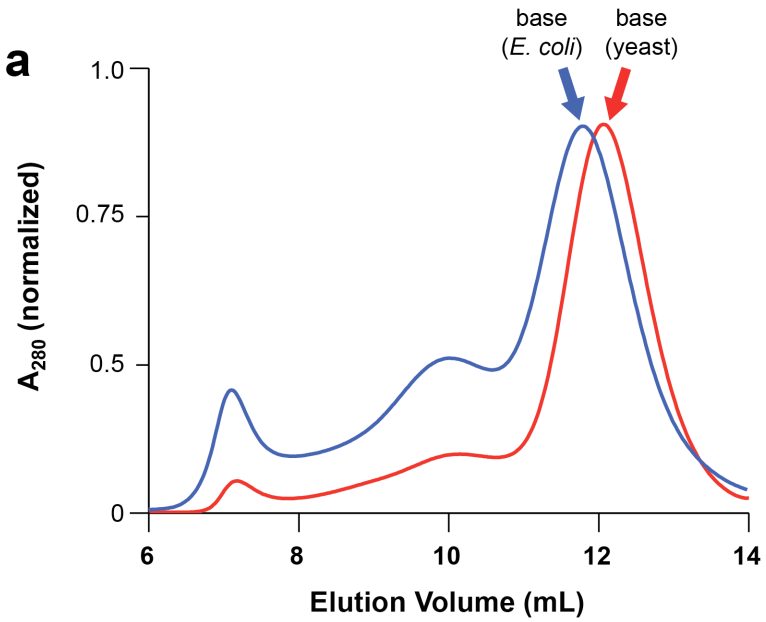
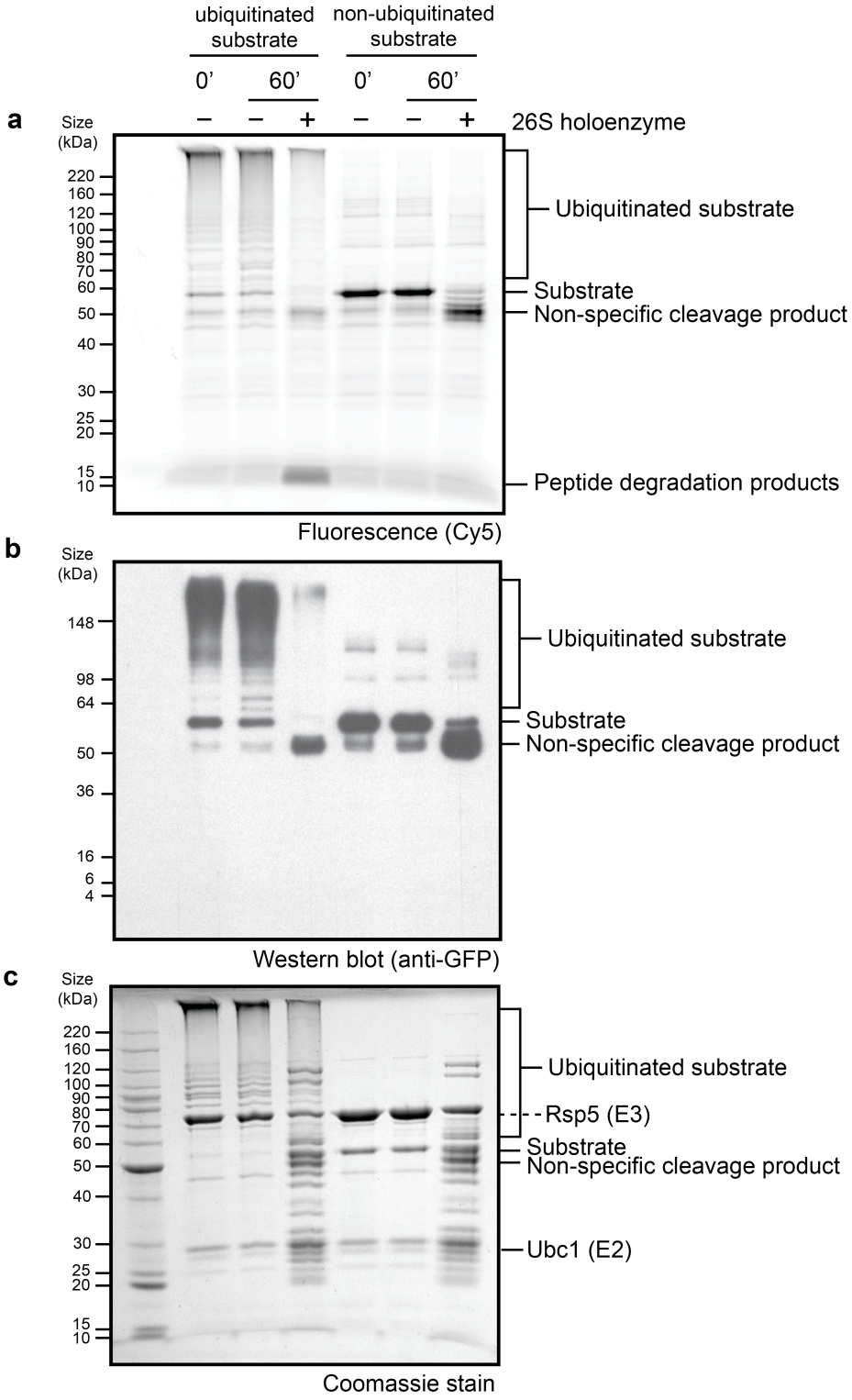
SUPPLEMENTARY FIGURES

Supplementary Figure 1:



Supplementary Figure 1. Purified recombinant base subcomplex resembles endogenous base.
Endogenous (red) and *E. coli*-expressed, recombinant (blue) base subcomplexes show similar elution profiles from a Superose6 size-exclusion column. The slightly smaller elution volume for recombinant base is attributed to the co-purification of proteasome-specific chaperones that stably associate with the complex when heterologously expressed in the absence of core particle in *E. coli* (see Fig. 1). The absorbance at 280 nm is normalized for comparison. For equal cell mass, recombinant base expression yields approximately 10-fold more protein than the purification of endogenous base from yeast.

Supplementary Figure 2:

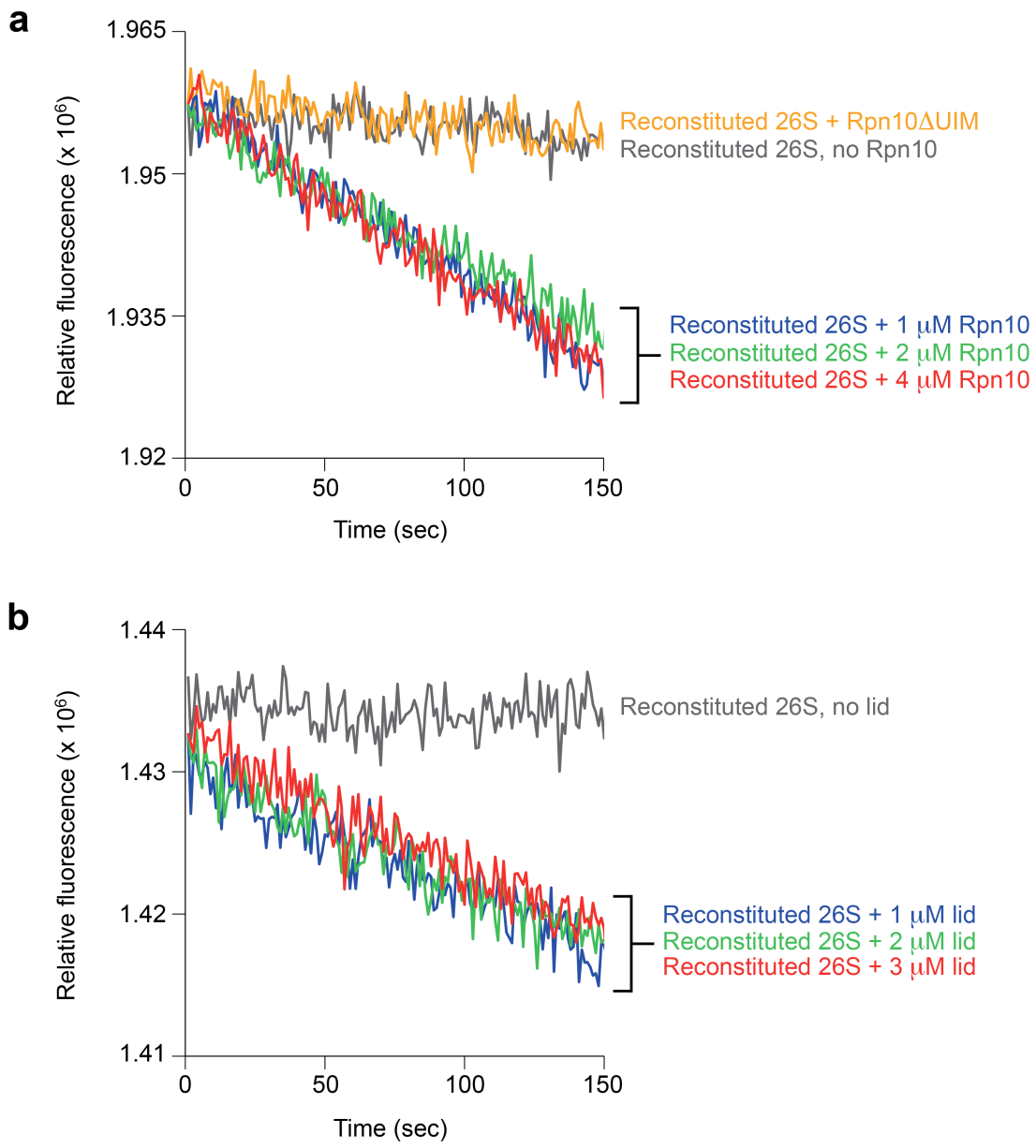


Supplementary Figure 2. Ubiquitinated GFP-fusion substrate is degraded by the 26S proteasome.

The model substrate, a green fluorescent protein (GFP)-titin^{V15P}-cyclin-PY fusion, was labeled at the N-terminus with Cy5 dye and subsequently modified with a polyubiquitin

chain *in vitro* using Uba1, Ubc1, Rsp5 and wild-type ubiquitin. The non-ubiquitinated substrate was prepared similarly except wild-type ubiquitin was omitted from the reaction. Degradation was assessed by incubating substrates with 26S holoenzyme purified from yeast in the presence of an ATP regeneration system at 30°C for one hour. Substrate degradation was then assessed by running samples on a SDS-PAGE gel followed by (**a**) fluorescence scanning to detect the Cy5-labeled substrate (670 nm band-pass 30 filter), (**b**) western blotting using an anti-GFP antibody, or (**c**) Coomassie staining for total protein. The fluorescence scan clearly shows the accumulation of small peptide degradation products only for the ubiquitinated substrate in the presence of holoenzyme. Some level of ubiquitin-independent partial cleavage of an unstructured region of the GFP model substrate was detectable in all three assays. Additional ubiquitination of the substrate was visible in the absence of holoenzyme both by fluorescence scan and anti-GFP western blot, which was not unexpected as the enzymes used for *in vitro* ubiquitination of the substrate were still present.

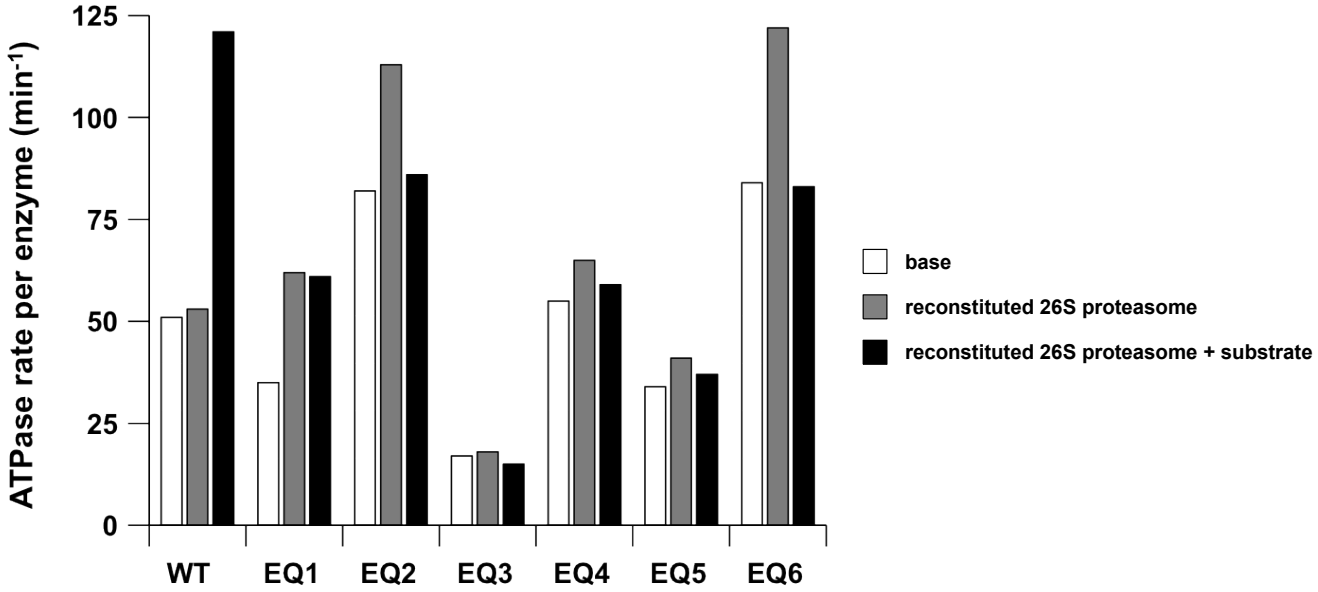
Supplementary Figure 3:



Supplementary Figure 3. Degradation rate for reconstituted 26S proteasome is not affected by excess amounts of lid or Rpn10.
Proteasomal degradation was monitored by the decrease in fluorescence of a polyubiquitinated GFP-fusion substrate (excitation 467 nm, emission 511 nm) upon incubation with reconstituted 26S proteasome. Degradation reactions contained limiting amounts of core particle (yeast) and saturating concentrations of base (*E. coli*-expressed), lid (yeast), and 1 μM Rpn10 (*E. coli*-expressed). To establish that excess amounts of free lid and Rpn10 did not interact with our ubiquitinated substrate and adversely affect the measured degradation rates, we added increasing amounts of (**a**) Rpn10 or (**b**) lid and observed that the degradation rate remained constant.

Supplementary Figure 4:

a

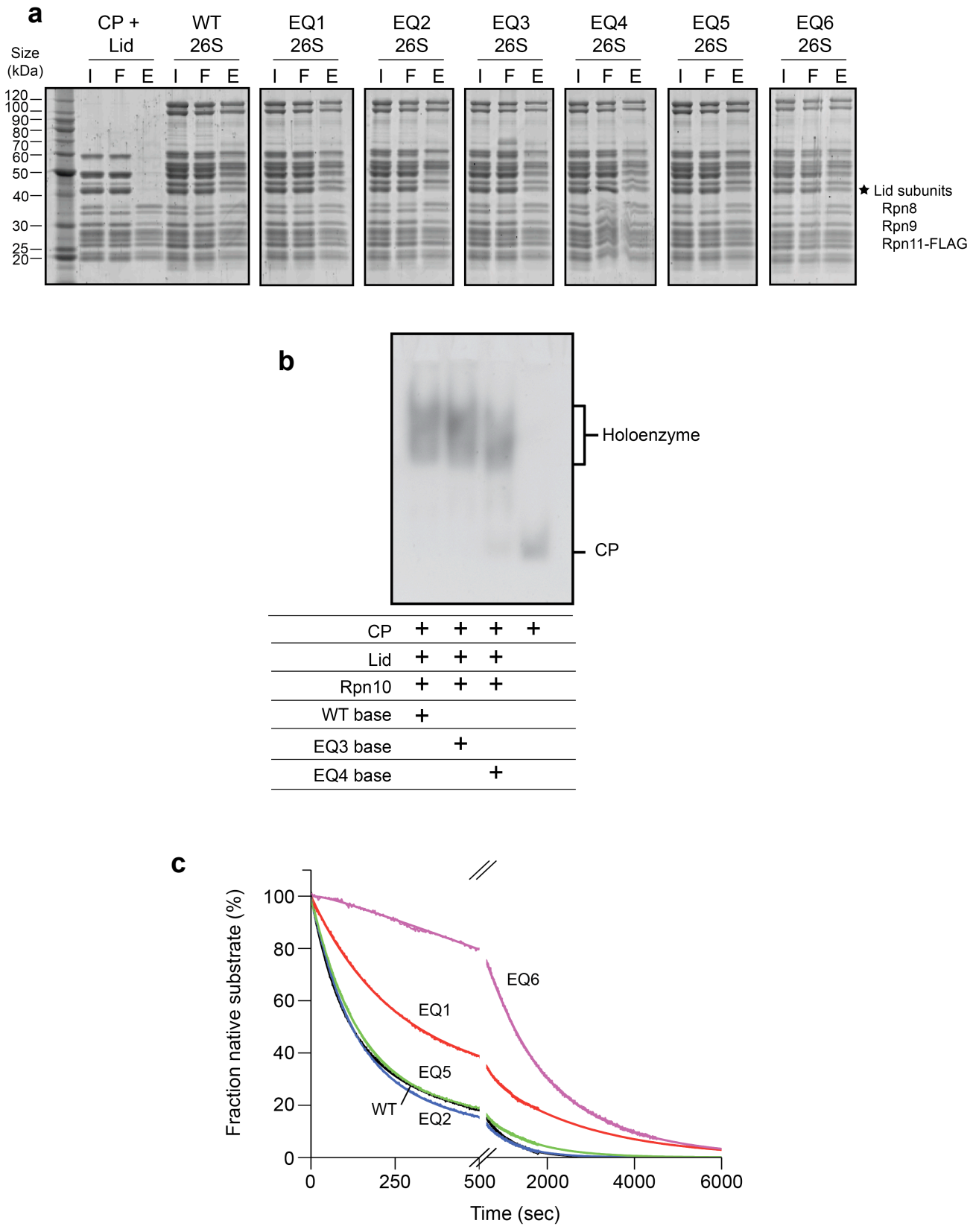


b

	basal ATPase rate (base)		**basal ATPase rate** (reconstituted 26S)		**working ATPase rate** (reconstituted 26S proteasome + ubiquitinated substrate)	
	min^{-1}	% WT	min^{-1}	% WT	min^{-1}	% WT
WT	51	100	53	103	121	237
EQ1	35	68	62	122	61	120
EQ2	82	161	113	221	86	169
EQ3	17	33	18	36	15	28
EQ4	55	108	65	127	59	116
EQ5	34	67	41	81	37	72
EQ6	83	164	122	240	83	162

Supplementary Figure 4. ATP hydrolysis rates for Walker-B EQ mutant base subcomplexes are not stimulated by ubiquitinated substrate.
Stimulation of ATPase activity by the base in the presence of ubiquitinated substrate was determined using a NADH-coupled ATPase assay. (**a**) Basal rates of ATP hydrolysis per enzyme (base hexamer) were determined both for base subcomplexes alone (white) and reconstituted 26S proteasomes containing the base variants (gray). Working ATPase rates were measured by adding ubiquitinated substrate to reactions containing reconstituted 26S proteasomes (black). (**b**) Table expressing the data from (a) in terms of ATP hydrolyzed per enzyme per minute and as a percentage of the rate observed for wild-type (WT) base alone. Errors for the ATPase assay were estimated to be ±10% of the WT mean value.

Supplementary Figure 5:



Supplementary Figure 5. Base variants with single-subunit Walker-B EQ mutations assemble into holoenzymes with varying degradation activities.
(**a**) Nickel affinity pulldown assay followed by SDS-PAGE analysis with Sypro Ruby staining to examine the assembly state of 26S proteasomes reconstituted with either wild-type (WT) or EQ base mutants. Untagged lid subcomplex was pulled down using His_6 tags on Rpt3 (base), Pre1 (core particle, CP) and Rpn10. Lanes for each sample are labeled I (input), F (flow through) and E (elution). Equivalent amounts of lid were observed for 26S proteasomes reconstituted with WT or EQ mutant base variants as indicated by the strong band containing lid subunits Rpn8, Rpn9 and Rpn11-FLAG (★). (**b**) Native gel analysis demonstrating that WT, EQ3 and EQ4 base variants are competent for assembly into 26S holoenzyme. Assembly of proteasomes was performed by incubating constituent subcomplexes with ATP, followed by native polyacrylamide gel electrophoresis as described in the methods. (**c**) Single-turnover degradation traces and curve fits for EQ base mutants. Degradation under single-turnover conditions was monitored by the decrease in fluorescence of 100 nM polyubiquitinated GFP-fusion substrate (excitation 467 nm, emission 511 nm) upon incubation with 2 μM 26S proteasome reconstituted with either WT base or EQ base variants. Proteasomes reconstituted with EQ3 or EQ4 base variants did not exhibit any measurable degradation even under single turnover conditions. Curves were best fit with a double exponential decay, likely reflecting degradation of two subpopulations of the substrate. These classes of substrate probably differ in the number or location of conjugated polyubiquitin chains but their affinity for the proteasome is expected to be similar.

SUPPLEMENTARY NOTES

Supplementary Note 1: Establishing Catalytic Mutations

Walker-B: "ATP-bound"

To fix subunits in a constitutively "ATP-bound" state[1], we examined mutations in the conserved Walker-B glutamate-aspartate motif by placing the same mutation in all six Rpt subunits simultaneously and characterizing the functional activities of the mutant base hexamers. We constructed a series of mutant hexamers containing single mutations of glutamate to glutamine (DN) or aspartate to asparagine (EQ) as well as the double DN EQ mutant. Rpt subunits carrying the DN mutation assembled properly into a hexamer that lacked ATPase activity. However, the DN mutation also had undesired deleterious effects, as this base variant was deficient in peptidase gate opening, even though a permanently ATP-bound hexamer is expected to interact with and maximally stimulate the core particle. The DN EQ double mutation resulted in an even stronger defect and prevented assembly of the ATPase hexamer, as indicated by the lack of an appropriate elution peak in size exclusion chromatography (unpublished data, R.B.). The EQ mutation supported proper base assembly, eluted at the appropriate volume from a size exclusion column, and was deficient in ATPase activity yet exhibited wild-type levels of peptidase stimulation (see Table 1). We deduced that the EQ mutation approximated an "ATP-bound" state and utilized this mutation for subsequent studies of the contribution of individual Rpt subunits to base activities (see Fig. 3, Table 1, Supplementary Fig. 4, and Supplementary Fig. 5).

Walker-A: "empty"

We required an appropriate mutation to induce a permanent empty state, either by preventing the conformational response of a subunit to ATP binding or by interfering with ATP binding itself. For some AAA+ enzymes, including ClpX from *E. coli*, mutation of the Sensor-II arginine has been shown to induce an empty-state conformation[1-3], yet the proteasomal clade of classic AAA+ enzymes lacks a conserved Sensor-II arginine. We therefore mutated the Walker A lysine to abrogate nucleotide binding altogether. First we mutated the lysine to either arginine (KR) or serine (KS) in all six Rpt subunits simultaneously to clearly establish the effects on base activity. The KR mutant hexamer exhibited activities in ATP hydrolysis and peptidase stimulation that were similar to wild-type base (unpublished data, R.B.), indicating that the KR mutation does not interfere with ATP binding of Rpt subunits. Conversely, the KS mutation resulted in severe assembly defects that prevented us from purifying the six-fold mutant base. This finding would be consistent with a completely empty-state hexamer and supports previous observations that assembly of the base is ATP dependent[4]. Furthermore, KS mutations in some individual Rpt subunits have been shown to interfere with cellular assembly of the 26S proteasome, compromise substrate degradation[5], and cause severe growth defects *in vivo*[6]. Preventing ATP binding simultaneously in all six subunits is likely more deleterious for ring assembly than placing the KS mutation in just a single Rpt at a time, as the assembly process may require only some subunits to fill with nucleotide. We therefore placed KS mutations in single Rpt subunits and characterized the functional activities of the resulting mutant base subcomplexes (see Fig. 5, Table 1). Only a mutation in Rpt2 caused extreme defects in base assembly**,** although all base variants with single KS mutations did exhibit some degree of misassembly.

Supplementary Note References:

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2. Joshi, S.A., Hersch, G.L., Baker, T.A. & Sauer, R.T. Communication between ClpX and ClpP during substrate processing and degradation. *Nat Struct Mol Biol* **11**, 404-11 (2004).
3. Song, H.K. et al. Mutational studies on HslU and its docking mode with HslV. *Proc Natl Acad Sci U S A* **97**, 14103-8 (2000).
4. Lee, S.H., Moon, J.H., Yoon, S.K. & Yoon, J.B. Stable incorporation of ATPase subunits into 19 S regulatory particle of human proteasome requires nucleotide binding and C-terminal tails. *J Biol Chem* **287**, 9269-79 (2012).
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6. Rubin, D.M., Glickman, M.H., Larsen, C.N., Dhruvakumar, S. & Finley, D. Active site mutants in the six regulatory particle ATPases reveal multiple roles for ATP in the proteasome. *Embo J* **17**, 4909-19 (1998).